AD_____

Award Number: W81XWH-09-1-0063

TITLE: Dynamic Testing of Signal Transduction Deregulation During Breast Cancer Initiation

PRINCIPAL INVESTIGATOR: Victoria Seewaldt, M.D.

CONTRACTING ORGANIZATION: Duke University
Durham, NC 27706

REPORT DATE: July 2012

TYPE OF REPORT: Revised Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
July 2012	Revised Final	1 July 2009 - 30 June 2012

4. TITLE AND SUBTITLE
Dynamic Testing of Signal Transduction Deregulation During Breast Cancer Initiation

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0063

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Victoria Seewaldt, M.D.

E-Mail: seewa001@mc.duke.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University
Durham, NC 27706

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Normal mammary gland homeostasis involves the coordinated regulation of cellular signaling networks. Current models suggest that that dysregulation of protein phosphorylation signaling occurs during breast cancer initiation. However, despite a wealth of mouse models and in vitro models of breast cancer, we have little prospective information on the biology of human breast cancer initiation. Here we aim to develop novel nanobiosensor technology to test live mammary epithelial cells taken directly from the breast of high-risk women for dysregulation of protein phosphorylation signaling. Under Specific Aim 1 will identify loss of normal protein phoshorylation signaling during breast cancer initiation. Under Specific Aim 2 we will develop targeted nanobiosensors to test real-time protein phosphorylation signaling in live Random Periareolar Fine Needle Aspriate (RPFNA) cells.

15. SUBJECT TERMS
Random Periareolar Fine Needle Aspirate (RPFNA); Reverse Phase Proteomic Microarray (RPPM)

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	9	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

We know little about the biology of breast cancer initiation in high-risk women. Here we have tested real-time protein phosphorylation signaling in live mammary cytology obtained from a cohort of well characterized high-risk women. Under Specific Aim 1 we tested for activation of high-risk signaling protein phoshorylation pathways during breast cancer initiation. Under Specific Aim 2 we are working to develop targeted nanobiosensors to test real-time protein phosphorylation signaling in live Random Periareolar Fine Needle Aspriate (RPFNA) cells. In our studies, we found that signaling pathways that are activated in aggressive breast cancer are also activated in pre-cancerous mammary epithelial cells.

The combined expertise, synergy, and resources of this collaboration provides a unique opportunity to investigate the origins of human breast cancer. The strength of this proposal is in its translational value, and clinical relevance. Technology developed in this proposal provide the basis for dynamic testing of live mammary epithelial cells and proof-of-principle for the development of functional imaging.

BODY:

Specific Aim 1 Identify loss of normal protein phoshorylation signaling during breast cancer initiation. Reverse Phase Protein Microarrays (RPPM) will quantitiate 50 total and phosphoproteins in duplicate from 5,000 microdissected Random Periareolar Fine Needle Aspirate (RPFNA) cells using published array technology. RPFNA cytology will span the range from normal cytology to hyperplasia, to atypia, to cancer. Phosphorylation signatures will be profiled relative to the degree of cytological abnormality. Supervised statistical analysis will test for dyregulation of protein phosphorylation networks.

Task 1: Prospectively obtain Random Periareolar Fine Needle Aspiration (RPFNA) cytology from a total of 325 women 1) 225 high-risk pre-menopausal women and 2) RPFNA from a minimum of 50 premenopausal women with DCIS and 50 premenopausal women with T1 breast cancer.

MILESTONES: ***Years 1-2:*** Perform 225 RPFNA in pre-menopausal high-risk women. We performed 279 RPFNA in pre-menopausal high-risk women. Of these, 167 were acceptable for microdissection and processing for Reverse Phase Proteomic Microarray (RPPM) analysis. These efforts are on-going and an additional 50 RPFNA will be performed over the next 6 months.

MILESTONES: ***Years 1-2:*** Perform RPFNA in 50 pre-menopausal women who are undergoing surgery for DCIS or breast cancer. We performed RPFNA on 295 women who were undergoing surgery for DCIS or breast cancer. Of these 295 samples, 201 were acceptable for microdissection and RPPM analysis in quadruplicate. We are currently finalizing this analyis.

Task 2: Perform Reverse Phase Protein Microarrays to test for protein phosphorylation patterns in a minimum of 200 informative RPFNA cytological specimens using protein array profiling.

MILESTONES: ***Reverse Phase Proteomic Microarray (RPPM).*** We have performed Protein Microarray profiling and quantitative analysis in 150 RPFNA specimens from our cohort. Our collaborator Chip Petricoin, PhD, validated and published Reverse Phase Protein Microarray (RPPM) to detect phosphoprotiein expression in 5,000 microdissected human cancer biopsy specimens. Over 302 phospho-specific antibodies and 60 total protein targets have been validated and brought to quantitiative array assay testing. Using RPPM, 50 endpoints in duplicate can be tested from RPFNA cytology. Sensitivity of the quantitiative array assay is in the attomole-zeptomole range. Internal dilution curves insure a high-dynamic calibration range.

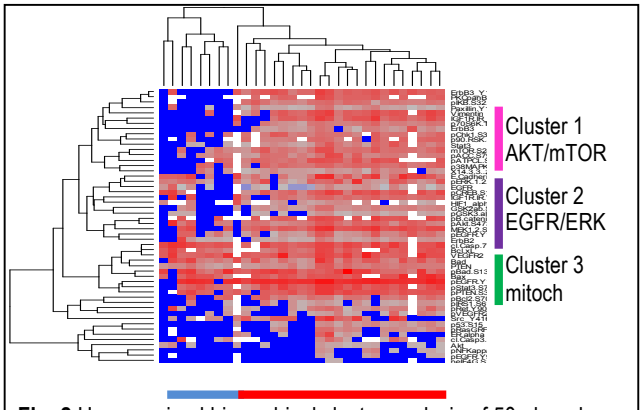

Fig. 2 Unsupervised hierarchical cluster analysis of 50 phospho-proteins in duplicate (n=39). Data sorts into low Masood (≤14 blue bar) and high Masood (>14 red bar). Three ER(-) expression clusters are identified Cluster 1 (pink bar), Cluster 2 (purple bar), Cluster 3 (green bar).

RPPM analysis of RPFNA cytology: We tested for the reproducibility of RPPM measurements for 53 individual proteins in quadruplicate. RPFNA samples from two independent test sets of 53 and 69 high-risk women described above (**Test Set #1** is depicted in **Fig. 2**). Five thousand epithelial cells were microdissected, solubilized, and tested by RPPM. Duplicate samples were prepared from microdissection of paired RPFNA aspirates. Fifty of 53 proteins tested by RPPM had an interclass correlation coeficient of agreement of >0.80. These 50 proteins included EGFR, EGFR-pTyr992, EGFR-pTyr1148, ErbB2, Erb2-pTyr1248, ErbB3, Erb3-pTyr1289, IGF1R, ERalpha, ERbeta-pSer118, IRS1-pSer612, MEK-pSer217/221, ERK-pThr202/Tyr204, HIF1alpha, VEGF, GSK3beta, AKT-pSer473, mTOR-Ser2448, 4EBP1-pThr37/46, eIF4G-pSer209, 70S6K-pThr412, FKHR-pSer256, IKB-pSer32-36, NFκB(p65)-pSer536, Bad-pSer136, Bcl2-pSer70, 14-3-3-ζ, p38MAPK, p90RSK, PTEN-pSer380, p53, p63, p21, Stat3, Stat5, Src-pTyr416, Fak-pTyr576, paxillin, beta-catenin-pThr42/Ser41, caveolin-1, vimentin, and E-cadherin**.**

Identification of high-intensity clusters: Three high-intensity ER(-) clusters were identified (**Fig 2**)

1) Cluster #1 (pink bar) activated Akt/mTOR/Src/ErbB2: Erb2pTyr1248, AKTpSer473, mTORpSer2448, 4EBP1pThr37/46, eIF4GpSer209, 70S6KpThr412, p38MAPK, p90RSK, 14-3-3-ζ, Src-pTyr416, Fak-pTyr576, Paxillin, Stat3, Vimentin.

2) Cluster #2 (purple bar) activated EGFR/MEK/ERK (**Fig. 3**) EGRF, EGRFpTyr1068, MEKpSer217/221, ERKpThr202/Tyr204, HIF1alpha, VEGF, GSK3beta-pTyr279/216, betacatenin-pThr42/Ser41.

3) Cluster #3 (green bar) mitochondrial survival proteins Bad, Bad-pSer137, Bcl2-pSer270, Bax, Bcl-xl, PTEN-Ser137 and HER2.

These studies have been published in:

1. Pilie P, Ibarra-Drendall C, Broadwater G, Barry W, Petricoin E, Liotta LA, Zalles C, Geradts J, Yu D, and Seewaldt VL. Protein Microarray Analysis of Mammary Epithelial Cells from Obese and Non-Obese Women at High-Risk for Breast Cancer. *Cancer Epidemiol Biomarkers Prevention*. 20:476-482, 2011 (cover article).

2. Ibarra-Drendall C, Barry W, Petricoin E, Yu D, Zalles C, and Seewaldt VL. Activation of protein network signaling during initiation of breast cancer in high-risk women. *Breast Cancer Res*, 2012.

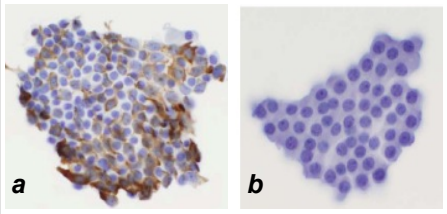

Fig. 4 Representative IHC vimentin expression in RPFNA atypical mammary epithelial cytology; (***a***) 3+ and (***b***) no expression.

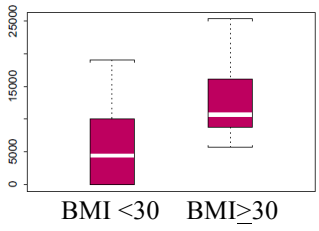

Fig 5. BMI vs. vimentin expression in atypical mammary RPFNA cytology.

The signaling network most highly activated (**Cluster #1 Fig. 2**) contained signaling proteins associated with the Warburg effect (AKT/mTOR/PI3K), insulin-signaling (pACC/IRS1), and epitheilial to mesenchymal transition EMT (IL6/Stat3/vimentin). Vimentin expression in mammary atypia was confirmed by IHC (**Fig. 4**). High Body Mass Index (BMI ≥30) correlated with high p-AKT and vimentin expression in atypical RPFNA cytology (**Fig. 5** and data not shown). These studies demonstrate our ability to identify signaling networks associated with the Warbug effect in atypical mammary cells from high-risk African American women.

MILESTONES: ***What is the frequency and distribution of protein phosphorylation in RPFNA as a function of Masood Cytology?*** Wilcoxion two-sample rank sum test will test for the distribution of protein phosphorylation in RPFNA as a function of Masood Cytology.

As above, we performed unsupervised heirachical clustering to test for the distribution of phosphoproteins as a function of Masood Cytology. We identified a set of phosphoproteins (Cluster 1) that were active in atypia (Masood >14) but not in non-atypia. These proteins include AKT/mTOR signaling proteins and also include vimentin, indicating a highly aggressive phenotype is women whose epithelial cells are undergoing epithelial to mesenchymal transition.

MILESTONES: ***What is the frequency of our RPPM proteomic signature in atypia?*** We will test for the frequency of our proteomic signature in atypia.
In two Test Sets, we identified that differential activation of AKT/mTOR-signaling was associated with cytological atypia (Cluster 1). We will now test a third test set in a larger set of samples to test for the frequency of our RPPM proteomic signature in atypia.

MILESTONES: ***What is the protein distribution in cancer?*** We commpared genomic and RPPM proteomic analysis of 152 pre-treatment primary breast cancers; 55 proteins were tested by RPPM including EGFR, EGFR-pTyr992, EGFR-pTyr1148, ErbB2, Erb2-pTyr1248, Erb3-pTyr1289, ERalpha, AKT-pSer473, mTOR-pSer2448, 4EBP1-pThr37/46, eIF4G-pSer209, 70S6K-pThr412, FKHR-pSer256, IKB-pSer32-36, NFκB(p65)-pSer536, IGF1R, HIF1alpha, VEGF, GSK3beta, MEK-pSer217/221, ERK-pThr202/Tyr204, Bad-pSer136, Bcl2-pSer70, 14-3-3-ζ, p38MAPK, p90RSK, PTEN-pSer380, p53, p63, p21, Stat3-pTyr694, Stat3-pSer727, Src-pTyr416, Fak-pTyr576, paxillin, betacatenin-pThr42/Ser41, vimentin, E-cadherin, IRS1-pSer612, ACC-pSer79, and IRS1-pSer612. Breast cancers underwent transcript profiling and were classified by genomic signature using the molecular subtypes established by Perou *et al.*[23]. Initially we found no correlation between the genomic subtypes and RPPM cluster analysis. Unsupervised hierachical cluster analysis identfied activated high intensity clusters containing Akt/mTor, Her2, and MAPK signaling proteins. As we accumulated samples and performed anlaysis, we found that there was a distinct correlation between 1) Triple-negative breast cancer and Akt/mTOR/IL6 activation, 2) Inflammatory signaling proteins and obesity, and 3) Her2 activation and HER2+ braest camcer. These results are in preparation for publication.

Specific Aim 2 Develop targeted nanobiosensors to test real-time protein phosphorylation signaling in live RPFNA cells. We will use a step-wise approach to develop targeted nanobiosensors. Phase 1: Our Preliminary Data suggest a key role for dysregulation of AKT-signaling during early mammary carcinogenesis. Dynamic testing of AKT signaling in mammary epithelial cells. Caspase-9 nanobiosensors will be developed and tested in defined systems before and after treatment with targeted AKT-pathway inhibitors. Nanobiosensor signaling will be standardized and quantitated using Reverse Phase Protein standard curves. Phase 2: Nanobiosensor technology will test RPFNA cytology obtained from high-risk women in our established cohort for dysregulation of AKT-phosphorylation and resistance to AKT-inhibitors. Nanobiosensor output will be quantitated and validated relative to quantitative microarray analysis. Phase 3: Guided by protein profiling in Aim 1 we will develop new targeted nanobiosensors

Task 1: Develop novel nanobiosenors to directly test for AKT-pSer-473 and AKT activity (Year 1).

MILESTONES: We developed caspase based nanobiosensors to be used in combination with an AKT inhibitor to test for AKT inhibition before and after treatment.

Task 2: Quantitative AKT phosphorylation signatures in 20 RPFNA samples to calibrate nanobiosensors.

MILESTONES: We initiated testing in RPFNA cytology from high risk women. This work is on-going. We have been successful in taking live cells from high-risk women, transporting them to the laboratory of Tuan Vo-Dinh, immobilizing the samples on a bio-friendly substrate, and then testing cells for caspase-9 activation with and without targeted AKT inhibitors. Cells were treated with 12 uM SH-6 for 15 minutes. Caspase-9 was detected in treated but not in untreated cells.

We have been able to rapidly test the samples, maintain viability, and accomplish testing within 1 hr of removal from a woman. Fig. 6 illustrates the insertion of the caspase-9 nanoprobe in live RPFNA cytology from high-risk women.

Task 3: Nanobiosensor technology is translated to test for pathway deregulation in RPFNA cytology obtained from 10 high-risk women with cytological atypia in our established cohort:

MILESTONES: ***Year 2:*** RPFNA samples from 60 women with cytological atypia will be tested using

nanobiosensors that target AKT-pSer473, AKT-pThr308, and total AKT; recruit 30 women by 12 mos and 30 additional women by 18 mos.

In parallel RPFNA cells will be treated in parallel with targeted AKT-inhibitors. RPFNA nanobiosensor output signals will be compared and validated relative to quantitative Reverse Phase Protein Microarray Profiling. We will develop calibration curves relative to quantitative microarray profiling and determine sensitivity, specificity, and reproducibility of nanobiosensors.

RPFNA cell clusters treated with AKT-inhibitors and tested using nanobiosensors will be marked, the slide fixed, and the cell cluster microdissected. Reverse Phase Protein Microarray Profiling will be performed and compared to the nanobiosensor signal.

KEY RESEARCH ACCOMPLISHMENTS:

1. Performed first proteomic profiling of mammary epithelial cytology in high-risk women.
2. Identified signaling pathways that are activated during the onset of breast cancer in high-risk women.
3. Performed the first nanobiosesnsor testing of live mammary epithelial cytology from high-risk women.
4. Tested for protein signaling in primary cancers.
5. Developed the biomarker data to drive a 300 person "personalized" medicine prevention trial.

REPORTABLE OUTCOMES:

Manuscripts

Scaffidi, J., Gregas, M., Ibarra, C., Seewaldt, V., Vo-Dinh, T. pH-sensitive nanoprobe for intracellular bioanalysis in living human cells. *Analytical Bioanalytical Chemistry*, 2009.

Pilie P, Ibarra-Drendall C, Broadwater G, Barry W, Petricoin E, Liotta LA, Zalles C, Geradts J, Yu D, and Seewaldt VL. Protein Microarray Analysis of Mammary Epithelial Cells from Obese and Non-Obese Women at High-Risk for Breast Cancer. *Cancer Epidemiol Biomarkers Prevention*. 20:476-482, 2011 (cover article). PMID: 21647677.

Ibarra-Drendall C, Barry W, Petricoin E, Yu D, Zalles C, and Seewaldt VL. Activation of protein network signaling during initiation of breast cancer in high-risk women. *Breast Cancer Res*, 2012. PMID: 21242333.

Ford A, Peterson B, Broadwater G, Ibarra-Drendall, Wilke L, and Seewaldt VL. Atypia in Random Periareolar Fine Needle Aspiration predicts short-term progression in high-risk women. Submitted *JNCI*, 2012.

Hoffman, A, Broadwater G, Ibarra-Drendall, Wilke L, Ford A, and Seewaldt VL. Glucose uptake in women at risk for breast cancer. Submitted *Cancer Epi Biomarkers Prev*, 2012.

Presentations

1. Ibarra, C., Troch, M, Broadwater, G, Wilke, L., Petricoin, E., Liota, L., and Seewaldt, V.L. Proteomic Signatures in Random Periareolar Fine Needle Aspirates. Plenary Talk, Breast SPORE, Washington, DC, 2010.
2. Ibarra, C, Petricoin, E., Broadwater, G., Wilke, L., Proteomic signatures differentiate atypia from normal mammary epithelial cells in high-risk women. Plenary Talk, AACR Prevention, Phil1adelphia, PA, 2010.
3. Seewaldt, V. Good breasts gone bad: breast cancer initiation in high-risk women. Platform talk, Gordon

Conference, Mammary Gland Biology, Salvia Regina, 2010.
4. Seewaldt, V. Breast cancer initiation in high-risk women. Keynote Speaker, EPA, 2011.
5. Seewaldt, V. Protein signaling in high-risk women. Era of Hope, Philadelphia, PA, 2011.
6. Seewaldt, V. Identification of high-risk signaling pathways. University of Colorado School of Medicine, Denver, CO, January, 2012.
7. Seewaldt, V. Activation of high-risk signaling pathways in women at risk for breast cancer. Bay Area PSOC, San Francisco, CA, February, 2012.
8. Seewaldt, V. Identification of high-risk signaling pathways. AVON Plenary Symposium, NYC, NY, March, 2012.
9. Seewaldt, V. Activation of high-risk signaling pathways in women at risk for breast cancer. City of Hope, Duarte, CA, March, 2012.
10. Seewaldt, V. Protein signaling in high-risk women. IABCR, Manchester, England, April, 2012.
11. Seewaldt, V. Identification of high-risk signaling pathways. Syndab Symposium, Atlanta, GA, May, 2012.

Career Development

1. Catherine Ibarra, Komen Career Catalyst, 2010, new faculty, Duke Univesrity.

2. Stacy Millon, PhD, 2010.

3. Molly Gregas, PhD, 2010, Postdoctoral Fellow, U. Toronto.

4. Julie Ostrander, K07 Award, 2010, new faculty, U. Minnisota.

5. John Scadiffi, Ph.D., new faculty, University of Miami

6. Sean Burrows, Ph.D., new faculty, Oregon State University.

7. Adria Surarez, third year medical student, Howard Hughes Research Year.

8. Abigail Hoffman, M.D., Susan G. Komen Postdoctoral Fellow.

9. Chris Sistrunk, Ph.D., K01 Minority Career Development Award.

Funding

1. Victoria Seewaldt, NIH/NCI R01CA155664-01, funded 07/2011.

2. Dihua Yu, Victoria Seewaldt, KG091020, funded 12/2009.

3. Julie Ostrander, K07, funded 08/2010.

4. Victoria Seewaldt, NIH/NCI R01CA158668-01A1, funded 04/2012.

5. Victoria Seewaldt, NIH/NCI 1R01CA170851-01, priority score 15, funding decision pending.

6. Victoria Seewaldt, NIH PSOC Pilot Award, Bay Area PSOC.

CONCLUSION:

This is the first report of proteomic profiling and nanobiosensor testing in live mammary epithelial cells from high-risk women. These tools are the building blocks that will allow us to track response to targeted agents in high-risk women. Since RPFNA can be performed repeatedly on women (this technique aspirates cells but does not biopsy), this allows us to sample cells before, during, after administration of a targeted agent. Since we trained our cooperative trial group (CALGB) to perform RPFNA, this allows us now to perform cooperative testing of targeted prevention in high-risk women. We recently obtained approval for a 300 person CALGB/ACOSOG/ALLIACE intergroup trial based on the technology developed in this proposal.

REFERENCES: None.

APPENDICES: None.

SUPPORTING DATA: None.